Letters to the Editor

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Reference

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2′,7′-Dichlorofluorescein is not a probe for the detection of reactive oxygen and nitrogen species

To the Editor:

In a recent issue of this *Journal*, Llacuna and colleagues [1] reported on the pivotal role of mitochondrial cholesterol accumulation and aggravated oxidative stress in the pathophysiology of ischemia–reperfusion (IR) injury in the fatty liver. Steatotic murine livers were subjected to 60 min of warm ischemia followed by a non-specified period of reperfusion. During reperfusion, part of the liver was incubated for 45 min with 200 μM 2′,7′-dichlorofluorescein (DCF) by means of superfusion and subsequently imaged by *in vivo* confocal microscopy, whereby DCF fluorescence was considered a direct measure of reactive oxygen species (ROS) generation. The data obtained with this technique served as the main premises for the conclusions related to intrahepatic oxidative stress.

However, DCF is not commonly used for the detection of ROS. Rather, the non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) is employed. $DCFH_2$-DA enters cells by passive diffusion. Inside the cytosol, esterases remove the acetate groups to form the non-fluorescent derivative 2′,7′-dichlorodihydrofluorescein ($DCFH_2$). Upon exposure to ROS as well as other oxidants such as reactive nitrogen species (RNS), $DCFH_2$ undergoes two-electron oxidation, resulting in generation of the highly fluorescent DCF (Fig. 1A). Accordingly, the extent of DCF fluorescence can be employed as a measure of the degree of oxidative/nitrosative stress, i.e., a state in which the generation of ROS/RNS outweighs the cell's antioxidative capacity [2].

Several experiments were performed to demonstrate the erroneous use of the probe. Firstly, the uptake of DCF by cultured human hepatocellular carcinoma (HepG2) cells was assessed. It was shown that DCF indeed accumulates in HepG2 cells at concentrations >20 μM (Fig. 1B and C).

Next, transcapsular penetration and hepatocellular uptake of DCF were evaluated *in vivo*. Superfusion of murine livers with DCF for 45 min resulted in marginal tissue penetration at best, with DCF fluorescence only exceeding background fluorescence intensity at the capsular interface (Fig. 1D, arrow).

To investigate whether DCF fluorescence increases upon oxidation, as reported by Llacuna *et al.* [1], DCF was incubated with Fenton reaction-derived hydroxyl radicals (·OH) [3]. A decrease in DCF fluorescence emission was observed following the addition of hydrogen peroxide (H_2O_2) to the DCF/iron(II) mixture, which led to the production of ·OH (Fig. 1E) [3]. This is consistent with an earlier report that identified DCF as a target for oxidation [4], but unequivocally revokes the conclusions of Llacuna and colleagues [1], who posited that DCF fluorescence intensifies as ROS production increases.

Conclusively, the proposition that DCF functions as an indicator for ROS production could not be confirmed. Rather, the opposite effects were observed, as evinced by the steep drop in DCF fluorescence upon ·OH generation. The data hence refute the oxidative stress-related conclusions by Llacuna *et al.* [1]. DCF is the end-product of a free radical species-specific fluorogenic probe (i.e., $DCFH_2$-DA/$DCFH_2$) rather than a probe for the direct visualization of ROS/RNS. Moreover, DCF appears to be oxidized to a non-fluorescent, yet unidentified, compound in the presence of ·OH, reducing the fluorescence intensity at 523 nm and thus reflecting an underestimated extent of intracellular ROS/RNS production.

Apart from the apparent misuse and subsequent misinterpretation of the results, two additional issues should be addressed. First, despite the fact that $DCFH_2$-DA and $DCFH_2$ are amongst the most common probes for the detection of ROS/RNS, little is known about the cellular behavior of these compounds. Although strongly stressed by some [5], studies that specifically investigate $DCFH_2$-DA and derivatives with respect to cellular kinetics, localization, and toxicity remain scarce to date [6,7]. The elusive intracellular kinetics and dynamics of $DCFH_2$-DA and derivatives could lead to wrongful use of the probe and hence misinterpretation of experimental results.

The second issue that exacts attention is the need to accurately describe and perform experimental procedures so as to ensure reproducibility and prevent the use of faulty methodologies, respectively. The incorrect use of materials or, for that matter, incorrect reporting on use of the proper materials (e.g., reporting on the use of DCF when actually $DCFH_2$-DA was employed instead), which can be found in various articles published in 2010/11 alone [8–10], could lead to widespread misapplication of the probe and the erroneous formulation of conclusions. This is especially important in regard to the data of Llacuna *et al.* [1], where it appears that a conclusion was drawn on the basis of incorrectly performed experiments.

Conflict of interest

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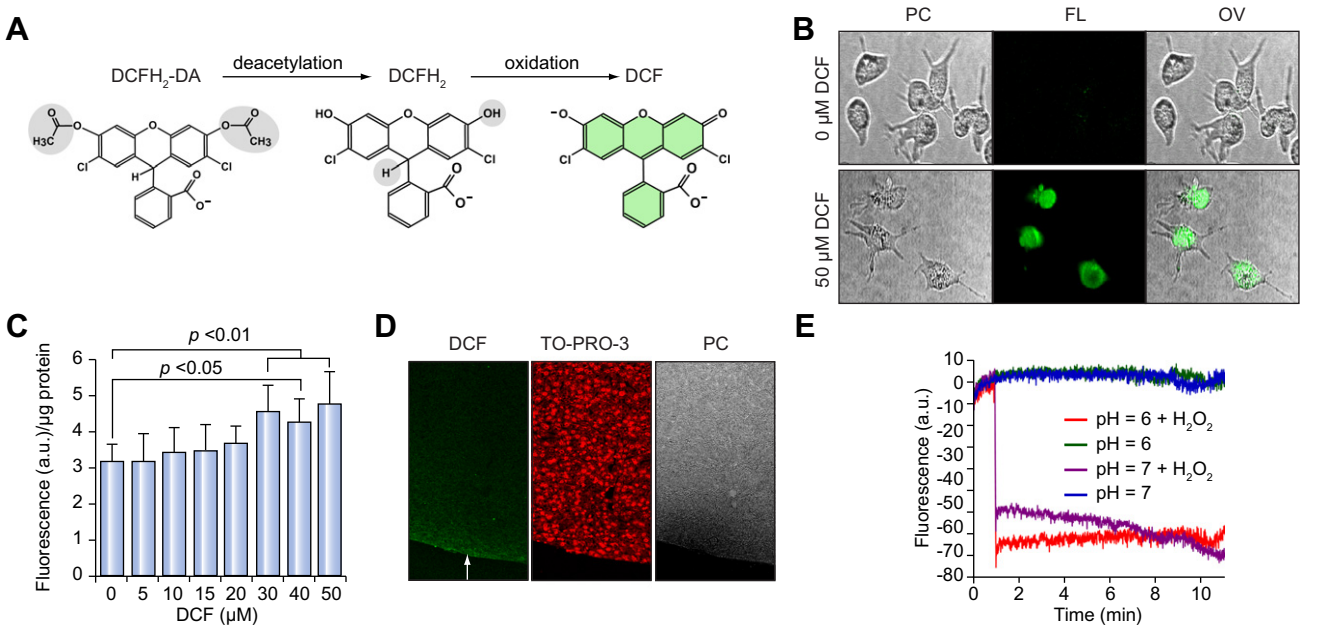

Fig. 1. DCF is not a probe for the detection of reactive oxygen and nitrogen species. (A) Chemical structures of $DCFH_2$-DA and derivates at physiological pH. $DCFH_2$-DA (non-fluorescent at 523 nm) is deacetylated (gray marquees, left panel) to $DCFH_2$ by cytosolic esterases. $DCFH_2$ (non-fluorescent at 523 nm) undergoes a two-electron oxidation (gray marquees, right panel) in the presence of ROS/RNS to yield DCF. DCF has an absorption maximum at 503 nm and an emission maximum at 523 nm in aqueous solution. (B) Uptake of DCF by HepG2 cells. Cells were grown on fibronectin-coated coverglass bottom dishes in William's E (WE) medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 0.1% insulin, and 0.2% hydrocortisone. Following 1 h incubation in PBS or 50 μM DCF in PBS at 37 °C, 5% CO_2, cells were washed and imaged by confocal microscopy at λ_{ex} = 496 nm and λ_{em} = 525 ± 25 nm. PC, phase contrast; FL, fluorescence; OV, overlay. (C) Quantification of DCF uptake by HepG2 cells as a function of DCF concentration. Cells were cultured on 24-well plates in supplemented WE medium as described above, washed, and incubated with 0–50 μM DCF in unsupplemented WE medium for 1 h at 37 °C, 5% CO_2. Following incubation, cells were washed and cellular DCF fluorescence was read with a microplate reader at λ_{ex} = 460 ± 40 nm and λ_{em} = 520 ± 20 nm. Fluorescence emission intensity was normalized to protein content. Statistical analysis was performed using a one-way ANOVA and Dunnetts's multiple comparison test following confirmation of normal distribution of data with a D'Agostino and Pearson omnibus test. (D) Superfusion of murine liver with DCF. The livers of adult male C57BL/6 mice (n = 3) were superfused with 60 μl of 200 μM DCF in PBS for 45 min. Subsequently, a punch biopsy of the superfused liver section was histologically processed and imaged by confocal microscopy. DCF fluorescence (λ_{ex} = 496 nm and λ_{em} = 525 ± 25 nm) is shown in green and TO-PRO 3 (λ_{ex} = 633 nm and λ_{em} = 670 ± 30 nm) was used as a clear stain (red fluorescence). (E) Oxidation-dependent DCF fluorescence kinetics. A cuvette containing 20 μM DCF and 2 mM Fe(II)SO_4 in a 0.2 M Na_2HPO_4/0.1 M citric acid buffer (pH = 6 or pH = 7) (1485 μl total volume) was placed in a temperature-controlled (20 °C) spectrofluorometer set to λ_{ex} = 500 ± 5 nm and λ_{em} = 523 ± 5 nm. During time-based acquisition, 15 μl of H_2O_2 (35.6 mM final concentration) or an equivalent volume of MilliQ was added at t = 1 min to the reaction mixture under continuous stirring. Addition of H_2O_2 resulted in the production of ·OH. Results represent the mean fluorescence emission values from n = 3 experiments per group. [This figure appears in color on the web.]

References

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Reply to: "2′,7′-Dichlorofluorescein is not a probe for the detection of reactive oxygen and nitrogen species"

To the Editor:
We received the letter by Rainiers *et al.* regarding our previous work [1], with mixed feelings. We are grateful to these authors for bringing to our attention the misspelling of the reagent used to monitor hepatic oxidative stress *in vivo* during ischemia/reperfusion (I/R) by two photon confocal imaging. However, we regret that 2′,7′-dichlorofluorescein appeared in the experimental procedures when in fact we actually used 2′,7′-dichlorofluorescein diacetate (Sigma, Catalogue No. D6883), also known as 2′-7′-dichlorodihydrofluorescein diacetate, a cell-permeable non-fluorescent probe that is de-esterified intracellularly and converted to the highly fluorescent 2′,7′-dichlorofluorescein upon oxidation, to ultimately detect ROS/RNS. We hope this unfortunate misspelling has not confused or misled researchers working in I/R liver injury.

We appreciate the effort by Rainiers *et al.* to confirm the inability of 2′,7′-dichlorofluorescein to detect ROS/RNS, although we believe investigators working in the field of oxidative stress for almost 30 years [2] know that 2′,7′-dichlorofluorescin diacetate or derivatives such as chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, Catalogue No. C6827) are the appropriate (reduced) substrates to monitor ROS/RNS, as reported in an outstanding number of papers, including ours [3–6].

While we apologize for the mistake, we regret the attempt of Rainiers *et al.* to invalidate the conclusions of our study based on the misspelling of the name of the probe, and not on the protocol used. The confocal microscopy data show a clear mitochondrial depolarization monitored with TMRM following I/R, particularly in mice exhibiting increased cholesterol loading. More important, the degree of hepatocellular damage monitored by transaminases and H&E staining increased substantially following I/R in mice exhibiting high hepatic cholesterol content, which was reduced by strategies that decrease cholesterol loading, such as statins. We believe our results report novel findings in the field of liver I/R injury, which may have important clinical implications for the near future. Finally, we agree with Rainiers *et al.* that attention should be paid to avoid mistakes or misspellings in research articles. In this regard, we welcome the initiative of some journals to include a detailed description of commercial products used in the study such as the catalogue or CAS number, which certainly will minimize future confusions.

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